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(Quarterly Progress Report)

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DEVELOPMENT OF AN ORALLY EFFECTIVE INSECT REPELLENT

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I. INTRODUCTION

During this report period additional compounds were analyzed for mosquito repellency by the electronic recording method, and further statistical analyses were performed as described in Report No. IITRI-L6021-4.

Work was initiated in two new areas, the trapping and the testing of effluents of animals for mosquito attractancy and the developing of a new approach toward elucidating the physiological basis of mosquito attraction.

II. ASSAY OF REPELLENTS WITH ELECTRONIC RECORDING SYSTEM AND STATISTICAL ANALYSIS

Table 1 was prepared in a similar fashion to that described in Report No. IITRI-L6021-4 and shows P + E, the discriminant function developed in that report.

Table 1

ANALYSIS OF MOSQUITO REPELLENCY OF VARIOUS COMPOUNDS BY ELECTRONIC RECORDING METHOD

Compound	Conc. on Mouse, mg	Time Displaced (P), %	Standard Displacement Distance (D) $\sqrt{\Sigma D^2/n}$	Mosquitoes Engorged (E), %	$\bar{P} + \bar{E}$, %	Significance at 95% Confidence Level[a]
N-Amyl succinimide	0.1	0	0	0		
	0.1	0	0	0		
	0.1	7.3	0.047	0	4.1	S
	0.1	5.3	0.118	3.8		
	0.01	35.6	0.326	4.0	39.6	S
2-Cyclohexyl-cyclohexanol	0.1	83	0.567	30.9	113.9	NS
N,N-Diethyl-m-isopropyl-benzamide	0.1	26.6	0.403	6.0		
	0.1	24.1	0.268	1.9	29.2	S
Cyclohexanol-2-phenyl	0.1	19.0	0.307	5.7		
	0.1	51.5	0.769	11.1	43.7	S
Methyl anthranilate	0.1	21.4	0.205	1.9		
	0.1	0	0	0		
	0.1	87.0	0.799	25.0	56.2	S
	0.1	75.6	0.329	15.0		
4-Cyclohexene-1,2-dicarboximide,N-propyl	0.1	0	0	0		
	0.1	0	0	0		
	0.1	36.8	0.486	5.6	15.6	S
	0.1	17.8	0.273	1.8		

Table 1 (cont.)

Compound	Conc. on Mouse, mg	Time Displaced (P), %	Standard Displacement Distance (D) $\sqrt{\Sigma D^2/n}$	Mosquitoes Engorged (E), %	$\bar{P} + \bar{E}$, %	Significance at 95% Confidence Level[a]
N,N-Diethyl-2,6-dimethyl benzamide	0.1	0	0	0	22.9	S
	0.1	0	0	0		
	0.1	24.2	0.328	6.2		
	0.1	50.8	0.450	10.3		
Succinamic acid,N,N-diethyl-sec-butyl ester	0.1	1.27	0.606	0	2.53	S
	0.1	0	0	0		
	0.1	24.0	0.328	0		
	0.1	0	0	0		
	0.1	0	0	0		
	0.1	0	0	0		
	0.1	0	0	0		
	0.1	0	0	0		
	0.1	0	0	0		
	0.01	51.0	0.450	2.3	37.6	S
	0.01	0	0	0		
	0.01	27.5	0.710	0		
	0.01	60.8	0.729	8.0		
2-Naphthol-1,2,3,4-tetra-hydro	0.1	0	0	0	0	S
	0.1	0	0	0		
	0.01	32.9	0.208	0	32.5	S
	0.01	28.2	0.654	3.8		
Propionanilide-N-butyl	0.1	0	0	0	0	S
	0.1	0	0	0		
	0.01	61.2	0.721	16.4	60.2	NS
	0.01	37.2	0.449	5.6		

Table 1 (cont.)

Compound	Conc. on Mouse, mg	Time Displaced (P), %	Standard Displacement Distance (D) $\sqrt{\leq D^2/n}$	Mosquitoes Engorged (E), %	$\bar{P} + \bar{E}$, %	Significance at 95% Confidence Level[a]
1,2-Cyclo-hexane-dicar-boximide-N-sec-butyl	0.1	0	0	0	0	S
	0.1	0	0	0		
	0.01	57.3	0.755	15.0	55.1	S
	0.01	28.9	0.423	9.0		
N,N-Diethyl benzamide	0.1	0	0	0	0	S
	0.1	0	0	0		
	0.1	0	0	0		
	0.1	0	0	0		
	0.01	50.1	0.331	5.4	27.8	S
	0.01	0	0	0		
Succinamic acid, N,N-dipropyl-sec-butyl ester	0.1	0	0	0	0	S
	0.1	0	0	0		
	0.01	34.6	0.506	3.1	54.9	S
	0.01	57.4	0.644	14.6		
Controls	-	100	1.082	41.8	128.2	-
	-	97.1	0.534	17.3		

[a]Significance was based upon an average of the various determinations at the same concentration level. S denotes a significant difference from the control at the 95% level of confidence. NS denotes that the difference is not significant at the 95% level of confidence.

The test for significant differences is developed as follows. The previous set of controls contained $n_c = 14$ observations. The mean for the variable $\bar{P} + \bar{E}$ was $\bar{X}_c$, or 113.6, with the estimated variance, S_c^2, equal to 1585.5. By using a t-test for significant differences of the means, relationship (1) can be stated.

$$\frac{\bar{X}_c - \bar{X}_i}{\sqrt{S_c^2\left(\frac{1}{n_c} + \frac{1}{n_i}\right)}} \geq t_{0.95(n_c - 1)} \tag{1}$$

where $\bar{X}_i$ is the mean of n_i observations for a test group. If relationship (1) is true, then the treated group is significantly different from the control group at a 95% confidence level. By using these statistics for the control group and $t_{0.95}(13) = 1.771$, Relationship 1 simplifies to

$$\frac{113.6 - \bar{X}_i}{\sqrt{1585.5\left(\frac{1}{14} + \frac{1}{n_i}\right)}} \geq 1.771 \tag{2}$$

Relationship 2 gives

$$113.6 - 70.5\sqrt{\frac{1}{14} + \frac{1}{n_i}} \geq \bar{X}_i \tag{3}$$

for a threshold condition with a significant repellent effect. Thus if $n_i = 2$, the mean for the sum $\bar{P} + \bar{E}$ must be less than or equal to 60.3%.

For the new data in which $n_c = 2$ and $\bar{X}_c = 128.1$, the previous estimate of variance $S_c^2 = 1585.5$, is used. The threshold condition becomes:

$$\bar{X}_i \leqq 128.1 - 70.5\sqrt{\frac{1}{2} + \frac{1}{n_i}}. \qquad (4)$$

For $n_i = 2$, the threshold is:

$$128.1 - 70.5\sqrt{\frac{1}{2} + \frac{1}{2}} = 57.6\% \text{ for the control group.}$$

For the three sets of variables measured, based on discriminant function analysis, the percent of time displaced (P) is the best single measure of repellency, the percent of mosquitoes engorged (E) is the next best measure, and the standard displacement distance (D) is the least reliable measure. The best variables for measuring repellency are P and E. These variables are the only ones required in these calculations.

Table 2 lists the threshold values for various numbers of tests (n_i) on a compound at a specific concentration. These values were derived from Relationship (4). If these values are exceeded, the experimental compound is not significantly different from the contro

Table 2

THRESHOLD VALUES FOR n_i

Number of Trials (n_i)	Threshold Value for Significant Difference
1	41.8
2	57.6
3	63.7
4	67.0
6	70.5
10	73.5

It is apparent from the results obtained with the electronic recording method that we have available a powerful screening instrument for potential mosquito repellents. This method promises to be of fundamental significance in the search for an orally effective insect repellent. Compounds that show repellency significantly above that of the controls can be applied in decreasing concentrations to test the concentration limit for repellency. Among the repellents tested during this and the last report period, some seem to be even more potent than diethyltoluamide (DEET) in this system. In this respect, succinamic acid, N,N-diethyl-sec-butyl ester; 2-naphthol-1,2,3,4-tetrahydro; N,N-diethyl benzamide; and possibly N-amyl succinamide appear to be outstanding. The numbers of tests on these compounds should be increased at the limits of effective concentrations to further substantiate these findings.

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A concentration of repellent that is just sufficient to prevent penetration of a host's skin by a mosquito is all that is necessary, regardless of whether a landing occurs. The elec-

rol.

tronic recording method offers a sensitive means by which such penetrations may be assessed.

We have microscopically observed the penetration of mosquito mouthparts into a transilluminated mouse's ear while simultaneous electronic recordings were made. The recorder responded only when the mosquito fascicle actually penetrated the skin of the ear.

If the proboscis merely touched the ear or quickly moved from one part of the ear to another in an apparent "searching" movement without penetration, the recorder did not respond. We are now sure that when the base line in the recording is displaced, the insect has actually penetrated the host's tissue.

III. TRAPPING AND TESTING OF ANIMAL EFFLUENTS FOR MOSQUITO ATTRACTANCY

The literature is replete with methods of testing insects for specific attractancy to various odors and effluents, and indeed in many cases specific attractants have been identified and chemically characterized. However, chemical substances that emanate from the host and specifically attract mosquitoes have never been identified.

The two definitely established components necessary for host-finding by mosquitoes are carbon dioxide and warmth. That carbon dioxide has an activating effect upon mosquitoes has been generally recognized and amply confirmed since the work of Rudolphs (ref. 1). Christophers (ref. 2) states, "Not only is there very strong evidence that warmth attracts A. aegypti in a very pronounced way in the urge to feed, but there has not been described in the literature any other attractive influence so active and characteristic in its results." We

nevertheless tested effluents of animals and humans for mosquito attractancy by using methods of collection that had not been attempted previously for this purpose.

To obtain samples for the attractancy test, 20 anesthesized mice were used. The mice were placed into a glass tube 30 in. in length and 2 in. in inside diameter. Dry carbon dioxide-free air was admitted into one end of the tube at a flow rate of 5 liters/min. The effluent air was passed into a Y-shaped tube which was immersed in a mixture of dry ice and acetone in order to condense and trap any volatiles emanating from the mice. After a 30-min collection period, the Y-shaped tube was removed and the open ends were immediately sealed with Teflon stoppers. A control sample was collected from the 30-in. tube before the mice were placed into it. The experimental and control samples were kept frozen until used.

Part of each sample was injected into an Aerograph model 204 two-channel detection system for separation and detection of the collected effluents. This device uses a flame-ionization detector as well as an electron-capture detector (electrons originate from a radioactive source) to analyze the effluents. The device traps gases on an oil-impregnated inert carrier, which is slowly heated to drive off the trapped gases at different temperatures. The gases then pass through the two detectors, and a chart recording shows the positions of gas evolution. Many components were seen in the effluents trapped from the mice.

The attractancy test was performed in a 30- x 12- x 12-in. glass tank covered with cheesecloth mesh. Approximately 500 female Aides aegypti mosquitoes were anesthetized by cooling and were placed into the glass tank. When the mosquitoes began to revive, the two previously collected samples were placed into the glass tank, opened, and dropped onto two watchglasses at opposite ends of the tank. The revived mosquitoes were carefully observed for 1 hr to determine whether the samples had any orienting influences upon their flight.

The sample containing the emanations from the mice showed no difference in attractance from the control sample, and, in fact, hardly a single mosquito landed upon or near either watchglass.

The experiment was repeated with nonanesthetized mice and a trap containing liquid oxygen, inserted in parallel with the dry ice-acetone trap. But even at the much lower temperature of liquid oxygen no effluent attractive to mosquitoes was detected. Similarly, human effluents, which were collected at 0°C, were not attractive to mosquitoes. We are now perfecting the system to collect human effluents at much lower temperatures. When these effluents are available, they will be tested.

Although the results were not surprising or unexpected, they confirmed the results of most other such experiments done in different ways.

The attractive properties of certain amino acids, especially lysine, have been described (ref. 3). However, it was later recognized that the attractiveness of lysine and other basic amino acids is largely due to trapped carbon dioxide bound in carbaminoyl complexes with the amino groups. The attractancy is in direct proportion to the amount of carbon dioxide bound (ref. 4), and these amino acids are no longer attractive when bound carbon dioxide is removed. However, it was also indicated that certain amino acids, such as tyrosine, cystine, and the prolines, may themselves attract mosquitoes (ref. 5).

On the fairly well-documented assumption that heat and carbon dioxide are the most important, if not the only, factors involved in attracting mosquitoes to their warm-blooded hosts, we have evolved a theory to explain how the interactions of heat and carbon dioxide operate in directing the mosquito to its host and the neurological events guiding this activity within the mosquito. If subsequent work proves this theory correct, a rational physiological and biochemical basis for interfering with host attraction can be established.

IV. A POSSIBLE MECHANISM OF HOST FINDING BY MOSQUITOES

For many years gamma aminobutyric acid (GABA) has been known to inhibit the transmission of afferent impulses across the myo-neural junction of crustaceans, such as crayfish (ref. 6). The close evolutionary relationship between crustacea and insects suggest that perhaps GABA or some GABA-like substance may also intermediate the transmission of impulses in the synaptic junction of insects, with special reference to mosquitoes. Indeed, GABA has been shown to be present in the nervous tissue of many mammalian and nonmammalian species. If GABA or a GABA-like substance (henceforth designated as GABA for convenience) does inhibit the transmission of impulses in the synapses of mosquitoes, how can carbon dioxide interact with this substance to cause host attraction in mosquitoes?

The work of Lipsitz and Brown (ref. 4) describes the high affinity that carbon dioxide has for lysine and the carbamino complex formed between carbon dioxide and this amino acid. Lysine and GABA have certain structural similarities in that they are both derivatives of carboxylic acids and have omega-amino residues, albeit lysine is a 6-carbon compound with an alpha-amino group and GABA is a 4-carbon compound with no alpha-amino group. Possibly GABA can form a carbaminoyl derivative with carbon dioxide just as lysine does. Indeed, alpha-amino butyric acid was one of the amino acids that shows a high attractance ratio for mosquitoes and significantly absorbs carbon dioxide (ref. 5).

If GABA is an inhibitor of transmission of impulses across synaptic junctions, perhaps when GABA is complexed with carbon dioxide, it can no longer function as an inhibitor and permits a much greater number of impulses to pass across the junction. In this case, carbon dioxide would act as an activator since it would deactivate the inhibitor. Carbon dioxide may thus indirectly act an an "irritant" to the mosquito.

In the presence of carbon dioxide, mosquitoes become activated and take to the wing. Initially, flight may be directionless and merely reflect an "attempt" by the mosquito to escape the "irritant." If, however, the carbon dioxide level in the mosquito's environment persists, the mosquito continues to fly, probably randomly. If the mosquito should happen to fly into a warm updraft of air, the heat may serve to uncouple the carbon dioxide from the GABA, and the mosquito may become somewhat less activated and more "confortable." That carbon dioxide can be driven from its complex with lysine by heating was shown by Lipsitz and Brown (ref. 4).

As the mosquito more closely approaches the source of heat, the GABA-carbon dioxide complex is increasingly cleaved, and the insect becomes less activated, and less irritated. If the heat source, however, originates from a warm-blooded mammal, as the mosquito approaches the heat more closely the carbon dioxide content of the air also increases. Therefore, though the GABA-carbon dioxide complex uncouples at a faster rate, it also forms

at a faster rate. A quickly reversing interplay of activation and inhibition now drives the mosquito directly to its host and eventuates in a landing. The warmth and the high carbon dioxide content at the surface of the host skin greatly accentuates the activity of the mosquito, and probing movements are one of the expressions of this heightened activity.

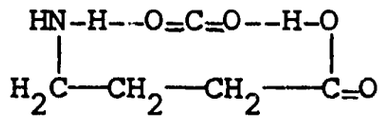
The effect of warmth is evident also in the need for warmth in the blood or in other fluid as a stimulus to feed. We have found, as have others (ref. 7), that when we feed A. aegypti through a membrane, it is necessary that the fluids behind the membrane be warm for feeding to be effective. Probing is an expression of a generalized increase of activity due to carbon dioxide. The tapping of a supply of warm blood and the mosquito's avidity for the source of the warmth may be an expression of the mosquito's attempt to decrease the irritant effects of carbon dioxide by bringing more warmth into its body. Blood, however, also contains carbon dioxide, so the chain of events leading to engorgement are only further stimulated.

Another mechanism may come into play when engorgement is complete. When the mosquito is fully distended with blood, the posterior pharyngeal value, which is involved in "swallowing," can no longer open to receive more blood because of the back-pressure of blood from the mosquito's abdomen. The valve is tightly shut and an outward pressure is exerted upon it. This outward pressure may cause the stimulation of other nerves that

are pressure receptors. The stimuli from these receptors may act antidromically to or inhibit afferent impulses in the synapse, which contains the GABA bound with carbon dioxide. This new set of impulses may effectively inhibit the afferent impulses which originally activated the mosquito, so that activator-irritant (carbon dioxide) becomes ineffective. Therefore, the mosquito withdraws its mouthparts and settles down to digest its meal. The difficulty of activating mosquitoes after a blood meal has long been recognized.

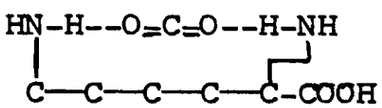
Although these assumptions now are purely speculative, if proven correct they would pave the way for a totally new method of approach to the development of mosquito repellents and indeed, of repellents for any other insect if the activating and deactivating components could be defined. If a carbon dioxide-binding fraction could be isolated from the mosquito and proven important in mosquito attraction in the way outlined above, experiments could be relagated from the insectary to the test tube. A more direct approach to the problem of developing an orally effective insect repellent thus can be achieved. As a first approximation, we possibly will look for a chemical substance that either binds GABA irreversibly or binds with greater affinity as a heat source is approached. The insect, not obtaining relief when heat is approached, then would not be attracted to its host.

Carbon dioxide could possibly bond with GABA in the following manner through a hydrogen-bonded carbon dioxide-GABA complex.



Molecular models show that such a structure is feasible and that the bond distances can accommodate this structure without internal molecular strain.

Lysine, an amino acid that is attractant to mosquitoes was also shown to be capable of combining with carbon dioxide (ref. 4). Molecular models show that a hydrogen-bond complex with carbon dioxide is possible also for lysine without undue molecular strain. This complex occurs in the following manner.



V. FUTURE WORK

During this next report period we will test the hypothesis outlined in Section IV. Several experimental approaches that we have conceived are outlined below. We hope that through these experiments we will obtain evidence as to whether this approach will be fruitful for pursuing in future work.

GABA and Carbon Dioxide. A glass tube will be packed with glass beads, and a solution of GABA will be added to the packed tube. The glass beads should increase the surface area of the GABA solution. A gas with a known carbon dioxide composition then will be passed through the tube. If GABA associates with carbon dioxide, the gas leaving the tube should have a decreased carbon dioxide concentration, which can be recorded on a carbon dioxide analyzer. Another tube with distilled water will be used as a control.

Inhibition of GABA-Carbon Dioxide Complex. If carbon dioxide complexes with GABA and if the complexing is indeed via hydrogen-bond formation, urea, guanidine, and other hydrogen-bond breaking substances should inhibit this complex formation. Urea will be added to the tube, and the effects of a hydrogen-bond breaker will be observed.

Effect of Temperature. Temperature also may have an effect upon carbon-dioxide-GABA complex formation. The tube experiment will be performed at different temperatures to observe these effects and to calculate the energy of activation for the complex formation.

Effect of pH. The effect of pH also can be studied via the tube experiment to discover the optimum pH conditions for the formation of GABA-carbon dioxide complexes.

GABA Titration Curves. A solution of GABA will be titrated, and the titration curve obtained will be compared with that of a solution of GABA with carbon dioxide bubbled through it. Complexing with carbon dioxide possibly shifts titration curves. This experiment can be performed also in the presence of urea.

GABA in Mosquitoes. To determine whether mosquitoes have GABA in their body composition, many mosquitoes will be collected, homogenized, and extracted with water; GABA is very soluble in water. The mosquito extracts will be chromatographed on paper in various solvent systems together with authentic samples of GABA to determine whether a spot on the paper migrates to the same position as GABA in various solvent systems. The spots can be developed with ninhydrin.

GABA-Like Substances in Mosquitoes. Possibly mosquitoes do not have GABA in their composition, but a GABA-like substance, which acts as an inhibitor of nervous impulse transmission across synapses. The inhibiting activity of the GABA-like substances may be overcome by complexing with carbon dioxide. Crude extracts will be placed into the tube and the carbon dioxide effluent will be analyzed. If carbon dioxide is trapped by the crude material, the components can be separately analyzed after chromatographic separation to discover which of these components complexes with carbon dioxide. Radioactive carbon dioxide, $C^{14}O_2$, can be used for this purpose.

The carbon dioxide complexing component can be further purified and identified chemically. Solvents other than water can also be used for extraction. The site at which carbon dioxide combining components exist in the mosquito can possibly be identified by using $C^{14}O_2$ tracers and autoradiographic techniques.

Extraction during Mosquito Life Cycle. The various states of mosquito development from larva to adult will be extracted to discover at what stage carbon dioxide combining components are formed. These components will probably be most abundant in the adult since in the adult the mechanism of host attraction is fully developed. Little or possibly none of these components may be extractible from larvae or pupae.

Carbon Dioxide Combining Capacity. The van Slyke apparatus will be utilized to quantitatively determine the degree of binding of carbon dioxide by the mosquito or by various extracts. The effects of urea on the carbon dioxide binding capacity of the extracts will also be determined. Formaldehyde should inactivate the binding of carbon dioxide by combining with the NH_2 groups of amines and thus eliminating one of the hydrogen-bonding sites on the complex. Analogues of GABA, such as gamma hydroxybutyric acid (GHBA), will also be tested for carbon dioxide combining capacity since GHBA is capable of hydrogen bonding with carbon dioxide. Such complex formation would substantiate our basic

hypothesis. GHBA has been tested in the myo-neural junction of crayfish and found to be a less effective inhibitor than GABA (ref. 8).

Effect of Heat. If our hypothesis is correct, mosquitoes that are placed or reared in a carbon dioxide free atmosphere should have no particular affinity for a heat source since they will not be in a state of activation. Comparison with controls should show whether mosquitoes bred in this way have a decreased affinity for heat. The carbon dioxide evolved from respiration may, however, affect the results of this experiment.

Crayfish Myo-Neural Junction. A crayfish myo-neural junction will be dissected out and immersed in a solution of GABA to observe inhibition of transfer of stimuli from the muscle to the nerve. The GABA solution will then be saturated with carbon dioxide, and we will observe whether impulses can pass across the synapse of the myo-neural junction in the presence of carbon dioxide.

The lines of investigation outlined are designed to establish basic mechanisms involving insect-host interactions. If these mechanisms can be elucidated, a rational approach to interference with the chain of events culminating in insect biting can be achieved.

VI. PERSONNEL AND RECORDS

The author wishes to acknowledge the technical assistance of Mr. Robert Fosler. The statistical analyses of the data were contributed by Mr. Merl Kardatzke.

All data are recorded in IITRI Logbooks C13755, C16400, C16417, and C16586.

Respectfully submitted,

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REFERENCES

1. Rudolphs, W., New Jersey Agr. Exp. Sta. Bull., Vol. 367, 1922.

2. Christophers, S. R., Aedes Aegypti (L.), Cambridge Univ. Press, p. 537, 1960.

3. Brown, A. W. A. and Carmichael, A. G., J. Econ. Entomol., Vol. 54, p. 317, 1961.

4. Lipsitz, E. Y. and Brown, A. W. A., Bull. Entomol. Res., Vol. 55, p. 395, 1964.

5. Roessler, P. and Brown, A. W. A., Bull. Entomol. Res., Vol. 55, p. 395, 1964.

6. Elliot, K. A. C. and Jasper, H. H., "Gamma-Aminobutyric Acid," Phys. Rev., Vol. 39, p. 383, 1959.

7. Bishop, A. and Gilchrist, B. M., Parasitology, Vol. 37, p. 85, 1946.

8. Edwards, C. and Kuffler, S. W., J. Neurochem., Vol. 4, p. 19, 1959.